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Aneuploidy Detection in Human Sperm Nuclei Using PRINS Technique

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Rapid and specific identification of chromosomes can be attained in situ using the PRimed IN Situ (PRINS) labelling technique. We have adapted this technique to mature human sperm in combination with a protocol for simultaneous decondensation and denaturation of sperm nuclei. This strategy allowed us to obtain double labelling of human spermatozoa in a <2-hr reaction. In the present study, we report the estimates of disomy for chromosomes 3, 7, 10, 11, and 17 on 64,642 spermatozoa from 2 normal males. The incidences of disomy ranged from 0.28–0.34%. There were no significant interindividual or interchromosomal differences in disomy rates. © 1996 Wiley-Liss, Inc.

KEY WORDS: PRINS, human sperm, chromosomes, aneuploidy

INTRODUCTION

Assessment of the aneuploidy rate in human gametes remains an important topic of interest and research, because nondisjunctions make a major contribution to the chromosomal abnormalities found in man.

In the last decade, direct information on the chromosomal constitution of human sperm has been obtained because of the introduction of the in vitro human sperm-hamster eggs fertilization system [Rudak et al., 1978; Martin et al., 1983]. This methodology has allowed the karyotyping of human sperm complements and has been applied in investigating various points concerning the occurrence and cause of chromosomal abnormalities in male meiosis [Martin and Rademaker, 1987; Pellestor et al., 1989]. However, this experimental method is labor-intensive and of little profit in terms of sperm karyotypes obtained. Recently, several laboratories have adapted interphase fluorescence in situ hybridization (FISH) technique on sperm in order to directly assess the incidence of disomy in human gametes [reviewed in Williams et al., 1993; Bischoff et al., 1994]. Chromosomal detection is usually performed with α-satellite repeat probes. The use of these satellite probes has some limitations; in particular, several chromosomes (5/19, 13/21, and 14/22) present high homology in their α-satellite DNA sequences, resulting in crosshybridization in FISH reaction [Waye and Willard, 1987; Lebo et al., 1992].

To overcome this problem, we have adapted the PRimed IN Situ (PRINS) labelling technique to human sperm. This technique is more efficient than the FISH procedure for discriminating among α-satellite DNA sequences [Koch et al., 1991; Pellestor et al., 1994]. We have generated primers specific for several human chromosomes and have established a dual-color PRINS protocol which distinguishes diploid and disomic sperm nuclei. In the present study, we report the results of aneuploidy detection for chromosomes 3, 7, 10, 11, and 17.

MATERIALS AND METHODS

Sperm Preparation

Sperm samples were obtained from 2 normal, healthy males, of proven fertility, ages 34 and 36 years, respectively. After liquifying at room temperature, freshly ejaculated samples were washed twice in phosphate buffered saline (PBS) by centrifugation (8 min at 2,000 rpm) and fixed for 1 hr in fresh fixative (3:1 methanol: glacial acetic acid) at −20°C. Sperm suspensions were then dropped onto clean microscopic slides and air-dried. Slides were aged 1–8 days at room temperature before use for PRINS reaction. Immediately before the reaction, the slides were denatured in 3 M NaOH at room temperature for 3–11 min, depending on their age.

Double PRINS Reaction

Specific oligonucleotide primers for α-satellite DNA of chromosomes 3, 7, 10, 11, and 17 were defined by comparing the α-satellite sequences of each chromosome to the consensus α-satellite sequence of human chromosomes reported by Choo et al. [1991]. The primer sequences were determined in the area with the

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most nucleotide divergences. The specificity of these primers was previously defined and tested on both metaphases and interphase nuclei [Pellestor et al., 1994, 1995a]. The sequences and the optimal technical conditions are given in Table I.

For each primer, a reaction mixture was prepared in a final volume of 50 μl, containing the oligonucleotide (50–200 pmol), 0.1 mM each of dATP, dCTP, and dGTP, 0.002 mM dTTP, 0.02 mM of biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim, Meylan, France), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% bovine serum albumin (BSA), and 2 units of Taq DNA polymerase (Boehringer Mannheim). The reaction was performed on the programmable temperature cycler Techne PHC-3 (Techne Corporation, Cambridge, UK), fit with a flat plate block. Prepared slides and coverslip were put on the plate block. Each reaction program consisted of two steps: 1) 12 min at annealing temperature, wherein slides were heated alone for 5 min to get them to the annealing temperature, after which the reaction mix was deposited on warm slides, spread with a coverslip, and heated 7 min; 2) 30 min at 72°C in order to allow nucleotide chain elongation. At the beginning of this second step, the temperature was automatically raised to 72°C. The first PRINS reaction was arrested by immersing the slides in a stop solution (500 mM NaCl/50 mM EDTA, pH 8) at 72°C for 3 min. Slides were then transferred from the stop solution to 1 × NT buffer (50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 100 μM DTT, and 150 μg/ml BSA) and washed twice at room temperature before being treated with a dideoxynucleotides mix (109 μM each of ddATP, ddCTP, ddGTP, and ddTTP, 4 μl 10 × NT buffer, and 2 U of Klenow enzyme) for 10 min at 37°C in order to block the free 3′-ends of the elongation fragments generated by the first PRINS reaction. This intermediate step prevented mixing of labelling. Slides were then passed in stop solution and washed twice in 1 × NT buffer at room temperature. The solution surplus was removed, and the slides were again placed on the plate block of the thermocycler. The second preheated PRINS reaction mix was then applied to the slides, which were heated 7 min at the annealing temperature of the second primer. The temperature was then automatically raised to 72°C, and slides were incubated 30 min. The reaction was stopped by immersing the slides in stop solution, and slides were transferred in 4 × SSC-0.5% Tween-20 at room temperature.

Detection

Biotinylated fragments were visualized using Texas red-avidin-DCS (5 μg/ml) (Vector Labs, Burlingame, CA), whereas detection of digoxigenin incorporated into synthesized products was done with antidigoxigenin-fluorescein antibody (20 μg/ml) (Boehringer Mannheim). Signals were simultaneously detected by incubating the slides 30 min with both Texas red-avidin and antidigoxigenin-fluorescein. The preparations were counterstained with propidium iodide (0.02 μg/ml) and 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI) (0.5 μg/ml) in the antifade solution Vectashield (Vector).

Scoring Criteria, Microscopic Analysis, and Statistical Calculations

Slides were analyzed by two independent observers, using a Leitz microscope equipped with a triple band-pass filter (DAPI:fluoresceinisothiocyanate (FITC): Texas red). Sperm nuclei were scored as haploid when they displayed two distinct signals in different colors (green and red). Nuclei were considered disomic when they displayed two similar signals, in size and intensity, separated by at least one domain diameter. The same criteria were applied for scoring diploid nuclei containing four distinct fluorescent spots (two green and two red).

Comparisons between mean frequencies of disomy were made using analysis of variance and standard deviation (SD). $P < 0.05$ was considered significant. χ^2 tests were performed to check for homogeneity in the rates of disomy for different chromosomes. Similar statistical analysis was carried out to compare diploidy frequencies.

RESULTS

The combination of 3 M NaOH treatment and the PRINS method allowed us to obtain an efficient labelling of sperm nuclei (mean, 98.5%) in a reaction of <2 hr. The time of optimal NaOH treatment depended on the age of the sperm preparation slides. The longer the slides were aged, the longer they needed 3 M NaOH treatment (1 day old, 3 min; 2 days old, 5 min; 4 days old, 7 min; 6 days old, 9 min; 8 days old, 11 min). Chromosome-specific primers were used in various combinations. In all cases, fluorescent signals were distinct and easily scorable. Examples of labelling are shown in Figure 1. Occasional size variations were observed (Fig. 1A) and were the direct consequence of variations in α-satellite DNA size.

The data obtained for the different primers are presented in Table II. A minimum of 10,000 sperm nuclei per chromosome primer were analyzed by two independent observers, leading to a total number of 64,642 scored spermatozoa. The mean frequency of disomy was 0.28% for chromosome 3, 0.31% for chromosome 7, 0.305% for chromosome 10, 0.345% for chromosome 11, and 0.32% for chromosome 17. There was no statistical difference ($P > 0.5$) in the frequencies of disomy among these five chromosomes.

TABLE I. Characteristics of Oligonucleotide Primers Used in the Present Study

Name	Locus	Chromosome location	Sequences	Annealing temperature (°C)	Optimal concentration (pM)
3c	α-satellite	3	5′ TGAGTTGAACACACACGTAC 3′	66	150
7c	α-satellite	7	5′ AGCGATTTGAGGACAATTGC 3′	56	100
10c	α-satellite	10	5′ ACTGGAACGCACAGATGACAAAGC 3′	63	200
11c	α-satellite	11	5′ GAGGGGTTTCAGAGCTGCTC 3′	65	200
17c	α-satellite	17	5′ AATTTCAGCTGACTAAACA 3′	51	200

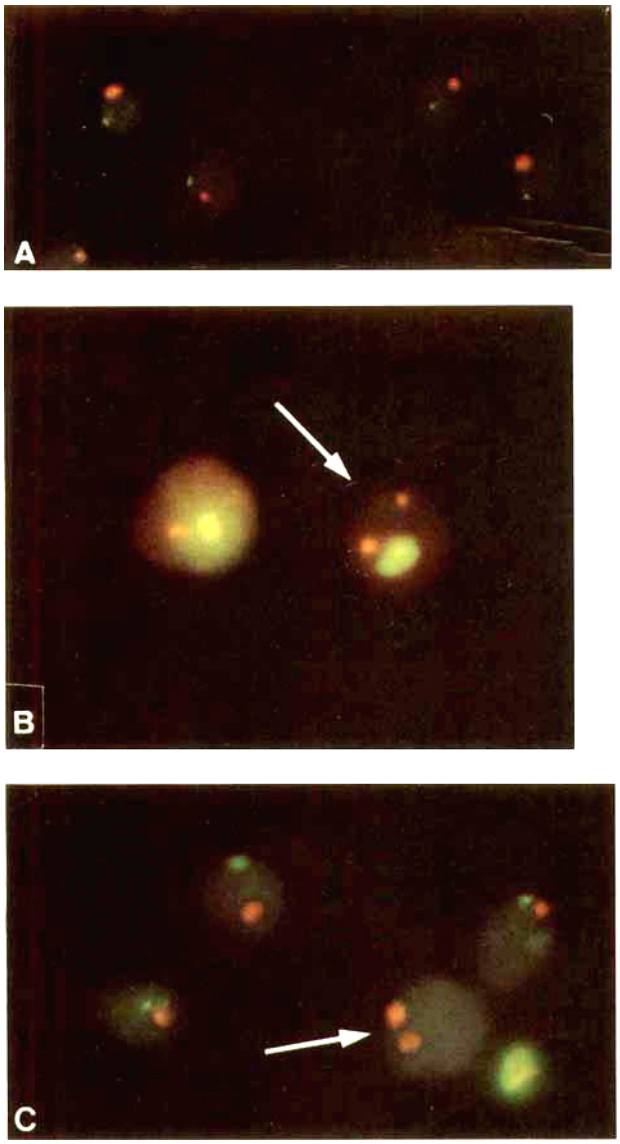

Fig. 1. In situ labelling of human spermatozoa by the PRINS technique. **A:** Normal haploid sperm labelled with α-satellite primers specific for chromosomes 3 (green spot) and 10 (red spot). **B:** Disomic spermatozoa (arrow) showing two distinct red spots (chromosome 17) and one green spot (chromosome 7). **C:** Sperm nuclei labelled with primers specific for chromosomes 10 (red spot) and 17 (green spot). Nucleus with arrow displays only two red spots, indicating either a double aneuploidy (a disomy for chromosome 10 and a nullosomy for chromosome 17) or a diploid sperm in which the labelling for chromosome 17 has failed.

The rate of diploid spermatozoa ranged from 0.11% in donor A to 0.25% in donor B, indicating a statistically significant difference ($P < 0.01$) between the two subjects in the frequency of diploid sperm nuclei.

TABLE II. Frequencies of Disomy and Diploidy for Chromosomes 3, 7, 10, 11, and 17 in Human Sperm

	Donor A	Donor B
Chromosome 3		
No. of nuclei scored	10,796	11,012
% of disomic nuclei	0.26	0.29
Chromosome 7		
No. of nuclei scored	11,005	11,124
% of disomic nuclei	0.33	0.29
Chromosome 10		
No. of nuclei scored	10,796	11,012
% of disomic nuclei	0.31	0.30
Chromosome 11		
No. of nuclei scored	10,204	10,501
% of disomic nuclei	0.37	0.32
Chromosome 17		
No. of nuclei scored	11,005	10,501
% of disomic nuclei	0.33	0.31
Mean % of diploidy	0.11	0.25

DISCUSSION

In the last few years, several studies have reported the use of FISH with repetitive probes for estimates of disomy rates in mature human sperm. On the basis of these data, no consensus concerning the incidence of nondisjunctions in spermatozoa is apparent. Martin and Rademaker [1994] pointed out that the estimates of aneuploidy in human sperm might vary >10-fold in different laboratories using FISH. This lack of uniformity could reflect the existence of intraindividual or intrachromosomal variability of the occurrence of nondisjunction [Robbins et al., 1993; Williams et al., 1993]. However, methodological variations seem to contribute significantly to such discrepancies in results. Early studies were performed using only one probe [Guttenbach and Schmid, 1991; Han et al., 1992; Martin et al., 1993]. This did not allow a real distinction between disomic and diploid nuclei and, consequently, may have led to misestimates of disomy frequencies. The use of stored sperm nuclei could also be incriminated. Several studies described storage of fixed frozen sperm before FISH analysis. Martin et al. [1994] demonstrated that storage of sperm nuclei affects both hybridization efficiency and the disomy estimate. Wyrobeck et al. [1993] demonstrated that α-satellite signals could split in human sperm nuclei, and that the lack of scoring criteria could consequently be a major reason for variations. The split of signals is directly linked to the in vitro decondensation of the sperm head. Because of the size of the probes, the difficulty in sperm analysis by FISH is to obtain efficient decondensation in order to increase the accessibility of DNA, without overswelling the sperm nuclei. The advantage of the PRINS strategy is the small size of primers used (18–30 nucleotides), which greatly facilitates their access to their genomic target. Thus, the PRINS reaction needs only moderate decondensation. We previously described a single-target PRINS protocol on sperm [Pellestor et al., 1995b]. This did not allow an accurate distinction between diploid and disomic spermatozoa. The present dual-labelling method permits us to overcome this problem. In addition, the NaOH pretreatment of sperm contributes to simplifying the protocol, since it allows the simultaneous decondensation-denaturation of sperm nuclei. Both the uniformity of the sperm labelling and the rapidity of the reaction demonstrate the efficiency of NaOH treatment in combination with PRINS.

The five chromosomes investigated displayed uniform disomy incidences (from 0.28–0.34%). Similar re-

TABLE III. Summary of Disomy Estimates (%) Reported for Chromosomes 3, 7, 10, 11, and 17

	Chromosomes				
References	3	7	10	11	17
Jackson-Cook and Haller [1991]					0.10
Han et al. [1992]					0.33
Bischoff et al. [1994]	0.34	0.04	0.19		0.09
Guttenbach et al. [1994]	0.31	0.31	0.32	0.34	0.31
Lu et al. [1994]		0.40			
Present study	0.28	0.31	0.30	0.34	0.32

sults were reported by Guttenbach et al. [1994] and, partially, by Bischoff et al. [1994] for the same chromosomes. Variations are only significant for chromosomes 7 and 17, with estimates ranging from 0.04–0.40% for chromosome 7, and from 0.09–0.33% for chromosome 17 (Table III). Due to the above reasons, it is difficult to determine if these variations reflect real variability in the occurrence of nondisjunction, or if they result from the methodological variations among different studies. Globally, reported rates were higher than those obtained by sperm karyotyping [Martin and Rademaker, 1990] or in some FISH studies for different chromosomes [Williams et al., 1993; Spriggs et al., 1995]. Possible explanations for these discrepancies could involve the small number of sperm complements analyzed per donor using the human sperm-hamster eggs fertilization system, or the fluctuations of hybridization efficiency of probe coktails according to the quality of sperm and methodological factors. Also, the possibility of interindividual variations of aneuploidy frequency cannot be excluded.

Uniform disomy rates, as reported in both the present study and some previous reports [Guttenbach et al., 1994; Miharu et al., 1994; Lu et al., 1994], support the assumption of an equal occurrence of nondisjunction among autosomal chromosomes in male gametes. This finding agrees with the data drawn from the cytogenetic analysis of human spermatozoa [Martin and Rademaker, 1990; Pellestor, 1991]. However, because of the existence of contradictory results in some FISH studies, this hypothesis needs to be confirmed for all chromosomes with a large sample of sperm from several individuals. In addition, comparative studies need to be performed with PRINS and FISH protocols in order to determine the efficiency of the various decondensation methods described, and to define a standard protocol.

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